Asian Herpetological Research 2017, 8(4): 262–268
DOI: 10.16373/j.cnki.ahr.170029

ORIGINAL ARTICLE

Effects of Constant versus Fluctuating Incubation Temperatures on Hatching Success, Incubation Length, and Hatchling Morphology in the Chinese Skink (*Plestiodon chinensis*)

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Abstract We incubated eggs of *Plestiodon chinensis* under five constant (24, 26, 28, 30, and 32 °C) and one fluctuating thermal regimes to examine the effects of constant versus fluctuating incubation temperatures on hatching success, incubation length, and hatchling morphology. The duration of incubation varied considerably among the six temperature treatments, whereas hatching success did not. The mean incubation length decreased as temperature increased in a nonlinear way, and increased as the thermal variance increased. Incubation temperature affected the body size (linear length and mass) and shape of hatchlings, with eggs incubated at 26, 28, and 30 °C producing larger and heavier hatchlings than did those incubated at 24 °C, 32 °C, or fluctuating temperatures. Our results showed that exposure of *P. chinensis* eggs to extreme temperatures for brief periods of time did not increase embryonic mortality and, in the fluctuating-temperature treatment, the thermal variance affected hatchling morphology more evidently than the thermal mean. Our results highlight the importance of the thermal variance in affecting embryonic development and hatchling morphology, and add further evidence that temperatures within the range of 26–30 °C are optimal for *P. chinensis* embryos.

Keywords Developmental plasticity, egg incubation, hatchling phenotype, scincid lizard, thermal variance, thermal mean

1. Introduction

The thermal environment that reptilian eggs experience during incubation can affect not only the rate of embryonic development but also a number of hatchling phenotypes that are sensitive to temperature, including body size, body shape, survival, immune response, locomotor performance, behavior, cognition, post-hatching growth, and even sex in species with temperature-dependent sex determination (Deeming, 2004; Valenzuela, 2004; Amiel *et al.*, 2014; Du and Shine, 2015). In most studies we can find that eggs were incubated at constant temperatures. However, the effects demonstrated in constant-temperature incubation do not reflect what really occurs in nature because eggs in natural nests are subjected to temperatures that are not constant but vary temporally and spatially (Cagle *et al.*, 1993; Overall, 1994; Ackerman and Lott, 2004; Booth, 2006). For example, reptilian embryos generally develop most successfully across a relatively narrow range (5–8 °C) of constant temperatures but can develop across a much broader range of temperatures in nature provided that daily exposure of eggs to extreme temperatures is brief (Birchard, 2004; Li *et al.*, 2013a). In recent years we have seen increasingly more studies where eggs are incubated under fluctuating temperatures to make results ecologically more meaningful (e.g., Ji *et al.*, 2003; Lin *et al.*, 2008; Neuwald and Valenzuela, 2011; Warner and

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Received: 21 April 2017 Accepted: 4 July 2017

Shine, 2011; Li *et al*., 2013a,b).

Studies of reptiles by incubation of eggs under fluctuating thermal regimes mimicking natural thermal conditions or in "outdoor incubators" have shown that fluctuating temperatures are important in some situations, but the importance varies among taxa, species, and even populations, and may affect some traits but not others (Neuwald and Valenzuela, 2011; Warner and Shine, 2011; Li *et al*., 2013a,b; Refsnider, 2013; Qu *et al*., 2014). In species where fluctuating temperatures influence embryogenesis differently than constant temperatures, that effect can result from the temperature fluctuation *per se*, or the fact that temperature fluctuation results in exposure of eggs to extreme temperatures that are likely to cause important phenotypic modifications (Li *et al*., 2013a,b). Thus, to address the question of whether temperature fluctuation has a role in influencing embryogenesis and resultant hatchlings, we need to study the temperature effects on a species-specific and trait-specific basis. Here, we incubated eggs of the Chinese skink (*Plestiodon chinensis*) under five constant and one fluctuating thermal regimes to examine the effects of constant versus fluctuating temperatures on hatching success, incubation length, and hatchling morphology. We chose this skink because it has a known range (24–32 °C; Ji and Zhang, 2001; Ji *et al*., 2002; Lu *et al*., 2014; Qu *et al*., 2014) of temperatures where successful embryonic development can take place, and is therefore well suited to the investigation testing the hypothesis that temperatures outside this range would adversely affect embryonic development and hatchling phenotype.

2. Materials and Methods

2.1 Animal collection and husbandry We collected 72 gravid females (86–115 mm snout-vent length, SVL) in mid-May between 2010 and 2011 from Lishui (28°27'N, 119°55'E; ~70 m elevation), Zhejiang, East China. Females were transported to our laboratory in Hangzhou, where 5–8 individuals were housed in each outdoor enclosure (length × width × height: 1.5 m × 1.5 m × 0.6 m) with a turf-covered substrate (~150 mm depth). Females could regulate body temperature by selectively using natural thermal flux. Mealworm larvae (*Tenebrio molitor*), house crickets (*Achetus domestica*), and water enriched with vitamins and minerals were provided daily. Females laid a single clutch of 9–24 eggs between 20 May and 26 June. During this period we checked the enclosures at least thrice daily for freshly laid eggs. Body mass and SVL were recorded for each post-oviposition female. All females were released at their sites of capture in late June in their sampling year.

2.2 Egg collection and incubation Eggs were collected and weighed less than 3 h post-laying, thereby minimizing water gain or loss between the egg and the substrate (Ji and Zhang, 2001). Fertilized eggs could be easily identified through the eggshell by visual inspection due to the presence of a reddish embryonic disc. Of the 1108 eggs collected, 972 were fertilized and could be incubated. One fertilized egg from each clutch was dissected to identify its Dufaure and Hubert's (1961) developmental stage, and the remaining eggs were individually placed into covered plastic jars (50 mL) with moist vermiculite at –12 kPa (Ji and Braña, 1999). In each sampling year eggs of the same clutch were assigned as equally as possible among treatments.

Eggs in the six treatments were either incubated in five incubators (Binder, Germany) set respectively at 24, 26, 28, 30, and 32 °C, or in a 400 mm × 300 mm × 200 mm chamber placed in a bush-covered backyard [hereafter the F (fluctuating-temperature) treatment], with three (the 24, 28, and 32 °C treatments) conducted in 2010 and three (the 26, 30 °C and F treatments) in 2011. We rotated jars at 2 d intervals to minimize the possible influence of thermal gradients; nevertheless, gradients in temperature within each incubator were trivial (0.5 °C), as verified by Tinytalk temperature loggers (Gemini Pty, Australia) placed inside jars. Substrate water potential was adjusted at 5 d intervals by weighing jars, and water was added to compensate for evaporative losses and water taken up by the egg. The F treatment was designed to address the effects of unpredictable and extreme temperatures. Thermal fluctuations in the chamber were monitored with a Tinytalk temperature logger inserted amongst the eggs and programmed to record temperature at an interval of 30 min throughout the experimental period (Figure 1). Daily mean temperatures ranged from 20.6 °C to 33.0 °C with a mean of 27.6 °C, daily minimal temperatures ranged from 19.1 °C to 30.3 °C with a mean of 25.2 °C, and daily maximal temperatures ranged from 21.0 °C to 37.3 °C with a mean of 28.6 °C. From earlier studies of *P. chinensis* we know that exposure of eggs to temperatures higher than 32 °C or lower than 24 °C for prolonged periods of time dramatically decreases hatchability (Ji and Zhang, 2001; Qu *et al*., 2014).

Incubation length was defined as the time interval between laying and pipping. Hatchlings were collected, weighed, and measured always less than 12 h post-hatching.

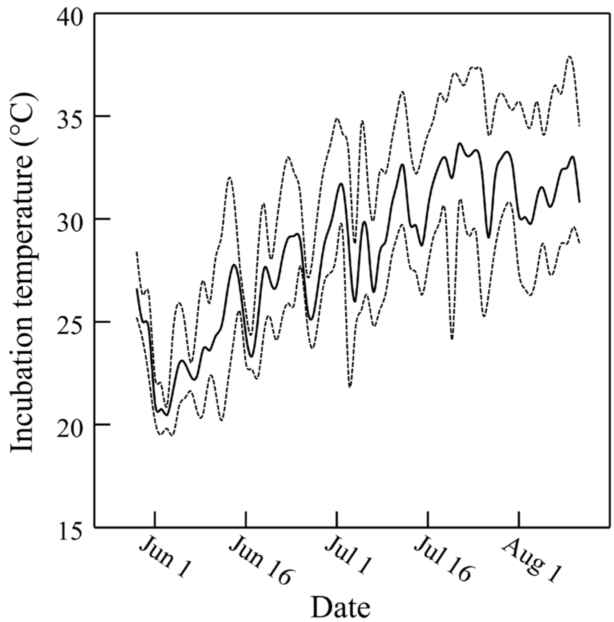

Figure 1 Temporal variation in daily minimal (lower dash line), mean (middle solid line) and maximal (upper dash line) temperatures experienced by *Plestiodon chinensis* eggs in the F treatment.

2.3 Morphological measurements We cooled hatchlings to approximately 5 °C, and then measured them with Mitutoyo digital calipers. Morphological measurements taken for each hatchling included SVL, tail length, head length (from the snout to the anterior edge of the tympanum), head width (taken at the posterior end of the mandible), forelimb length (humerus plus ulna), and hindlimb length (femur plus tibia).

2.4 Statistical analyses We used log-likelihood ratio test (*G* test) to examine whether eggs incubated under different thermal regimes differed in hatching success. We used linear regression analysis to examine whether an examined variable was related to egg mass at laying. We used partial correlation analysis to examine correlations between the selected pairs of variables while holding the remaining variables constant. We used one-way ANOVA (for egg mass at laying and incubation length) and one-way ANCOVA (for hatchling morphological variables) with egg mass at laying as the covariate to analyze data. Multiple comparisons were performed using Tukey's test for the variables that differed among treatments. Prior to parametric analyses, data were tested for the homogeneity of variances using the Bartlett's test, and for the normality of data using the Kolmogorov-Smirnov test. All statistical procedures were performed in Statistica 8.0 (StatSoft; Tulsa, OK, USA), and statistical significance was assumed at $P < 0.05$. Values are presented as mean ± standard error (SE) and range.

3. Results and Discussion

Freshly laid *P. chinensis* eggs varied in mass from 0.51 to 0.84 g, with a mean of 0.67 g. Embryonic stages at laying ranged from Dufaure and Hubert's (1961) Stage 30 to 36, with a mean stage of 32.6. Mean values for egg mass and embryonic stage at laying did not differ among the six temperature treatments (ANOVA: both $P > 0.612$). The mean egg mass was within the range (0.66–0.70 g) of values reported for the same population of *P. chinensis*, and the range and mean of embryonic stages at laying were also similar to the values (30–36, with a mean of 32.4) reported for the populations (Lin and Ji, 2001; Lu *et al*., 2012, 2014; Qu *et al*., 2014). Given that data collection in this and previous studies last for 15 years (from 1997 to 2011), these consistencies suggest that the mean and variability of egg size and embryonic stage at laying are remarkably constant from year to year in the Lishui population of *P. chinensis*.

Hatching successes varied from 83% (194/234) in the 32 °C treatment to 89% (149/167) in the 28 °C treatment, with an overall mean of 86% (Table 1). Eggs assigned to different treatments did not differ in hatching success ($G = 3.99$, $df = 5$, $P > 0.50$). Eggs of *P. chinensis* have been incubated at constant and fluctuating temperatures for various topics (Ji and Zhang, 2001; Ji *et al*., 2002; Du *et al*., 2005; Lu *et al*., 2012, 2014; Qu *et al*, 2014). Available data show that hatching successes are high (> 82%) across the temperature range from 24–32 °C, although exposure of eggs to temperatures higher than 30 °C or lower than 26 °C for prolonged periods of time increase energetic costs during the embryonic stage and/or result in the production of smaller hatchlings (Ji and Zhang, 2001; Ji *et al*., 2002; Lu *et al*., 2014; Qu *et al*., 2014). In the present study, eggs in the F treatment had the possibility of exposure to extreme temperatures as low as 19 °C and as high as 37 °C. However, hatching success (~86%) was high in the treatment. This provides further evidence that exposure of eggs to extreme temperatures for brief periods of time may not necessarily increase embryonic mortality in reptiles (Lin *et al*., 2008; Ji *et al*., 2003; Lu *et al*., 2009; Li *et al*., 2013a,b).

Within each treatment incubation length was independent of egg mass (linear regression analysis: all $P > 0.20$). The mean incubation length differed among the six treatments ($F_{5,\ 581} = 2913.90$, $P < 0.0001$). Within the range of constant temperatures from 24–32 °C incubation length decreased as temperature increased in a nonlinear

way, with the mean length shortened by 8.1, 7.7, 3.6, and 1.4 d for each 2 °C increase from 24 to 32 °C (Table 1). This pattern of thermal dependence of incubation length is consistent with earlier studies of turtles (Ji *et al*., 2003, Du *et al*., 2007, 2010), lizards (Ji and Braña, 2001; Lin *et al*., 2007) including *P. chinensis* (Ji and Zhang, 2001; Lu *et al*., 2014), snakes (Ji and Du, 2001; Lin *et al*., 2005; Lin *et al*., 2010), and crocodiles (Piña *et al*., 2003; Charruau, 2012).

A partial correlation analysis on data from the F treatment showed that incubation length was negatively correlated with thermal mean ($r = -0.91$, $t = 27.97$, $df = 157$, $P < 0.0001$) but was positively correlated with thermal variance ($r = 0.35$, $t = 4.67$, $df = 157$, $P < 0.0001$). The positive correlation between incubation length and thermal variance suggests that *P. chinensis* is among reptiles including turtles (Ashmore and Janzen, 2003; Les *et al*., 2007, 2009; Du *et al*., 2009), lizards (Hao *et al*., 2006; Andrewartha *et al*., 2010; Du and Shine, 2010; Li *et al*., 2013a), and snakes (Lin *et al*., 2008; Patterson and Blouin-Demers, 2008; Lu *et al*., 2009; Löwenborg *et al*., 2012) where eggs at constant temperatures hatch earlier than those at fluctuating temperatures with the same thermal mean. The reason for why eggs at constant temperatures hatch earlier lies in that the decelerated developmental rates at temperatures below a given level cannot be equally offset by the accelerated developmental rates at temperatures above that level, because the rate at which embryonic development increases with temperature becomes increasingly smaller as temperature increases (Li *et al*., 2013a,b; Lin *et al*., 2008; Lu *et al*., 2009; Löwenborg *et al*., 2012; Qu *et al*., 2014). It is worth noting that constant incubation temperature does not always results in early hatching. For example, in species with temperature compensation in embryonic development, eggs at constant temperatures may hatch no earlier than those at fluctuating temperatures with the same thermal mean (turtles: Du and Ji, 2003; Ji *et al*., 2003; Li *et al*., 2013b; lizards: Andrews *et al*., 2000; Du and Ji, 2006; Lin *et al*., 2007; Li *et al*., 2012; snakes:

Table 1 Hatching success and incubation length (expressed as mean ± SE and range) of *Plestiodon chinensis* eggs incubated under six thermal regimes.

Thermal treatments (°C)	# incubated eggs	Hatching success (%)	Incubation length (d)
24	209	86.4 (181/209)	39.9 ± 0.1 (33.0–44.0)
26	73	88.5 (65/73)	31.8 ± 0.2 (29.3–34.0)
28	167	89.0 (149/167)	24.1 ± 0.1 (19.0–26.0)
30	102	86.6 (88/102)	20.5 ± 0.1 (17.8–22.7)
32	234	83.0 (194/234)	19.1 ± 0.1 (14.2–21.6)
F	187	85.6 (160/187)	25.9 ± 0.2 (20.0–36.0)

Table 2 Descriptive statistics, expressed as mean ± SE and range, for egg mass at laying (initial egg mass) and wet body mass and morphology of *Plestiodon chinensis* hatchlings from eggs incubated under six thermal regimes. *F* values and significance levels of one-way ANOVAs (for initial egg mass) or ANCOVAs (for the remaining variables) with initial egg mass as the covariate are indicate in the table. Treatments with different superscripts differ significantly (Tukey's test, α = 0.05, a > b > c > d).

	Thermal treatments						*F* values and significance level
	24 °C	26 °C	28 °C	30 °C	32 °C	F	
N	181	65	149	88	194	160	
Initial egg mass (mg)	647.9 ± 6.0 462.0–898.0	650.2 ± 6.7 498.7–763.5	648.9 ± 6.6 482.0–883.0	642.5 ± 6.4 497.0–821.0	640.8 ± 6.0 436.0–865.0	650.0 ± 6.2 447.0–924.0	$F_{5, 831} = 0.39$, $P = 0.853$
Wet body mass (mg)	614.3 ± 6.1 370.0–839.0	640.5 ± 8.9 420.5–770.2	633.0 ± 6.2 44.0–845.0	651.6 ± 6.5 479.1–817.6	607.5 ± 5.6 413.0–808.0	610.6 ± 5.9 429.0–808.0	$F_{5, 830} = 20.72$, $P < 0.001$ 24^{bc}, 26^{ab}, 28^{b}, 30^{a}, 32^{c}, F^{c}
Snout-vent length (mm)	29.7 ± 0.1 26.5–32.9	29.8 ± 0.2 25.6–32.9	30.0 ± 0.1 26.7–32.5	29.8 ± 0.1 25.1–33.0	29.0 ± 0.1 24.5–32.7	29.6 ± 0.1 24.8–32.5	$F_{5, 830} = 14.23$, $P < 0.001$ 24^{ab}, 26^{ab}, 28^{a}, 30^{ab}, 32^{c}, F^{bc}
Tail length (mm)	35.9 ± 0.2 29.9–41.8	37.5 ± 0.3 29.0–41.1	37.7 ± 0.2 30.0–44.3	37.3 ± 0.3 26.6–49.6	35.6 ± 0.2 27.3–44.1	36.6 ± 0.2 29.6–43.8	$F_{5, 830} = 15.33$, $P < 0.001$ 24^{c}, 26^{ab}, 28^{a}, 30^{ab}, 32^{c}, F^{b}
Head length (mm)	7.3 ± 0.1 6.7–8.0	7.3 ± 0.1 6.8–8.0	7.4 ± 0.1 6.8–7.9	7.4 ± 0.1 6.8–7.8	7.1 ± 0.1 6.6–7.7	7.1 ± 0.1 6.6–7.6	$F_{5, 830} = 12.20$, $P < 0.001$ 24^{bc}, 26^{abc}, 28^{a}, 30^{ab}, 32^{d}, F^{cd}
Head width (mm)	5.0 ± 0.1 4.5–5.4	5.0 ± 0.1 4.5–5.5	5.1 ± 0.1 4.5–5.5	5.0 ± 0.1 4.5–5.4	4.8 ± 0.1 4.2–5.3	4.9 ± 0.1 4.4–5.4	$F_{5, 830} = 27.32$, $P < 0.001$ 24^{b}, 26^{ab}, 28^{ab}, 30^{a}, 32^{d}, F^{c}
Forelimb length (mm)	6.0 ± 0.1 5.0–7.0	6.2 ± 0.1 5.4–6.9	6.2 ± 0.1 5.2–6.9	6.1 ± 0.1 5.3–6.5	5.9 ± 0.1 4.9–6.7	5.9 ± 0.1 4.9–6.6	$F_{5, 830} = 19.17$, $P < 0.001$ 24^{b}, 26^{ab}, 28^{a}, 30^{b}, 32^{c}, F^{c}
Hindlimb length (mm)	7.2 ± 0.1 6.1–8.1	7.4 ± 0.1 6.6–8.1	7.5 ± 0.1 6.4–8.5	7.3 ± 0.1 6.4–7.7	7.0 ± 0.1 5.8–8.1	7.1 ± 0.1 6.0–8.1	$F_{5, 830} = 24.80$, $P < 0.001$ 24^{b}, 26^{a}, 28^{a}, 30^{ab}, 32^{d}, F^{cd}

Table 3 Results of partial correlations between a selected pair of variables, with the effects of a third variable held constant, based on data from *Plestiodon chinensis* hatchlings (N = 160) from eggs incubated at fluctuating temperatures. NS: not significant; *: P < 0.05; **: P < 0.01; ***: P < 0.001.

	Thermal mean	Thermal variance
Body mass	-0.30^{NS}	-0.71^{***}
Snout-vent length	-0.18^{NS}	-0.56^{**}
Tail length	-0.27^{NS}	-0.42^{*}
Head length	0.086^{NS}	-0.29^{NS}
Head width	-0.21^{NS}	-0.62^{**}
Forelimb length	0.091^{NS}	-0.082^{NS}
Hindlimb length	-0.23^{NS}	-0.36^{NS}

Chen and Ji, 2002; Ji *et al*., 2007a).

All examined morphological traits were positively related to initial egg mass (linear regression analysis: all P < 0.05) and differed among the six treatments (Table 2). Eggs incubated at temperatures from 26 °C to 30 °C generally produced larger and heavier hatchlings that also had larger heads, limbs and tails than those incubated at 24 °C, 32 °C, or fluctuating temperatures (Table 2). In hatchlings from the F treatment we found that: (1) none of the examined morphological traits was correlated with thermal mean; and (2) body mass, SVL, tail length and head width were negatively correlated with thermal variance (Table 3). Temperature fluctuations during incubation have no role in affecting the phenotype of hatchlings in a wide range of reptile taxa including turtles (Du and Ji, 2003; Ji *et al.*, 2003; Li *et al*., 2013b), lizards (Hao *et al*., 2006; Andrewartha *et al*., 2010; Li *et al*., 2013a), and snakes (Chen and Ji, 2002; Lin and Ji, 2004; Lin *et al*., 2008; Lu *et al.*, 2009). An earlier study of *P. chinensis* also shows that temperature fluctuations during incubation have no role in affecting the phenotype of hatchlings as long as eggs are not exposed to extreme temperatures for prolonged periods of time (Qu *et al*., 2014). However, studies of reptiles also exist in which incubation temperature fluctuation has been found to affect at least one phenotypic attribute. For example, incubation temperature fluctuation affects locomotor performance of hatchlings in the smooth soft-shelled turtle *Apalone mutica* (Ashmore and Janzen, 2003), the northern grass lizard *Takydromus septentrionalis* (Du and Ji, 2006), the multi-banded krait *Bungarus multicintus* (Ji *et al.*, 2007a), and the black ratsnake *Elaphe obsolete* (Patterson and Blouin-Demers, 2008). In two turtle species with temperature-dependent sex determination, *Trachemys scripta* (Les *et al.*, 2007) and *Chrysemys picta* (Paitz *et al.*, 2010), incubation temperature fluctuation affects offspring sex ratios. It is worth noting that in none of these studies could differences in hatchling phenotype be attributable to incubation temperature fluctuation with much certainty because thermal fluctuations may result in exposure to extreme temperatures that may modify hatchling traits. For example, hatchling checkered keelbacks (*Xenochrophis piscator*) from eggs incubated at 30 °C and temperatures fluctuating from 20 °C to 36 °C are smaller than those from eggs incubated at 24 °C and 27 °C (Lu *et al*., 2009). The explanation for this difference is that prolonged exposure of *X. piscator* eggs to the temperature of 30 °C or higher can result in the production of less developed and thus smaller hatchlings (Ji *et al*., 2001). Thermal fluctuation also has no important role in inducing phenotypic variation in viviparous reptiles such as the many-lined sun skink *Eutropis multifasciata* (Ji *et al*., 2007b), the toad-headed lizard *Phrynocephalus vlangalii* (Wang *et al*., 2014), and the short-tailed pit viper *Gloydius brevicaudus* (Gao *et al*., 2010) where gravid females maintain body temperatures as stable as possible through more precise thermoregulation not because temperature fluctuation *per se* have any role in modifying offspring phenotype but because the range of temperatures optimal for embryonic development is narrow (Ji *et al*., 2007b; Gao *et al*., 2010; Wang *et al*., 2014).

In summary, our results highlight the importance of the thermal mean and variance in affecting embryonic development and hatchling morphology in *P. chinensis*. From this study we know the following. First, incubation length decrease as temperature increases in a nonlinear way, and increases as the thermal variance increases. Second, incubation temperature affects the body size and shape of *P. chinensis* hatchlings, with eggs incubated at 26, 28, and 30 °C producing larger and heavier hatchlings than did those incubated at lower or higher temperatures. Third, exposure of *P. chinensis* eggs to extreme temperatures as low as 19 °C or high as 37 °C for brief periods of time does not necessarily increase embryonic mortality. Fourth, the thermal variance affects hatchling morphology more evidently than the thermal mean. Our results add further evidence that temperatures within the range from 26 °C to 30 °C are optimal for *P. chinensis* embryos.

Acknowledgements The Forestry Bureau of Lishui City provided the permit for capturing lizards in the field. Our experimental procedures complied with the current laws on animal welfare and research in China. We thank Guo-Hua Ding, Jian-Fang Gao, Zhi-Hua Lin, and Jing Yang for assistance during the research. For funding, we thank the National Natural Science Foundation of China (31470471

and 30670281), the Zhejiang Provincial Natural Science Foundation (LY17C030003), and the Priority Academic Development Program of Jiangsu Higher Education Institutions.

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